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Human Anti-Breast Cancer Peptide

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1. Introduction:

We have synthesized a peptide that inhibits the growth of ER+ human breast cancers growing as xenografts in immune-deficient mice, including those that are resistant to tamoxifen (Tam) (1). This nine amino acid peptide, cAFPep (previously referred to as COP), is derived from alpha-fetoprotein (AFP) which itself has anti-estrotrophic and anti-breast cancer activity (2). It has been shown that the peptide does not act like Tam or any other known agent currently used to treat ER+ breast cancer (1;2). Although the anti-breast cancer activity of cAFPep and its precursors is well-documented (1;3;4), neither the mechanism of its action nor that of AFP has been fully elucidated (1;2).

Both cAFPep and its parent molecule AFP would not be expected to freely cross the plasma membrane because of their charge and size. Cell surface receptors have been isolated and characterized for peptide molecules such as oxytocin, vasopressin, and somatostatin and its analogs (5-7), and for proteins such as insulin and insulin-like growth factor (8). The receptors for these molecules act as mediators of signal transduction (5-7;9). Therefore it is reasonable to assume that this peptide interacts with (a) cell surface receptor(s) to mediate its anti-breast cancer activity. While it is possible that the AFP-derived peptide will interact with a receptor for AFP, this remains to be determined. Receptors for AFP have been studied in various undifferentiated cell lines, but have not been completely characterized and no sequence information is available. Isolation and characterization of the receptor for cAFPep will elucidate the first step in the mechanism of anti-oncotic action of this peptide.

During the first year of this training grant, peptides have been synthesized for use in the affinity chromatography (AC) procedure to isolate proteins that have an affinity for the AFP-derived peptides. These peptides have been linked to an affinity column matrix, and the procedure for isolation of breast cancer cells that bind to the peptide has been optimized. A 70 kDa protein was retained by columns coupled to both active and inactive peptides, including peptides with a disrupted pharmacophore, indicating putative non-specificity in the strategy designed to isolate the cAFPep receptor. The second year investigations studied this 70 kDa protein and the isolation strategy in more depth. Multiple proteins with an approximate molecular weight of 70 kDa were seen when affinity column eluents were separated by electrophoresis. One of these proteins which showed qualitative specificity for the AFP-derived peptide was identified as heat shock protein 70 (HSP70). Since HSP70 interacts with the estrogen receptor (ER), investigations during the third year focused on further examining the interactions between HSP70 family members, cAFPep, and the ER in human breast cancer cells.

2. Body:

Research Accomplishments (Final)

A. Isolation of AFPep-specific Cell-Membrane Binding Protein(s) by Affinity Chromatography:

1. **Identification of the pharmacophore of the AFP-derived peptide.** During the first year of this training grant (see May 2003 report), peptides were synthesized for use in an affinity chromatography procedure to isolate cellular proteins from human breast cancer cells which had an affinity for the AFP-derived peptide. These peptides included linear and cyclized AFP-derived peptides, and various control peptides which showed no inhibitory activity in an *in vivo* assay that measured inhibition of E₂-dependent growth. Before the cyclized AFP-derived peptide could be coupled to an affinity column it was necessary to determine where the pharmacophore of the peptide was located and to engineer an attachment site into this cyclic peptide to facilitate coupling to the affinity column matrix. Amino acid substitutions were rationally designed and the peptide analogs synthesized and their

inhibitory activity determined. Both conservative and non-conservative substitutions of key amino acids which abolished this inhibitory activity indicated where the pharmacophore of the peptide was located. During the second year, this work culminated in a publication in the *Journal of Peptide Research* (10). Substitution of a lysine for methionine at a site distal to the pharmacophore in cAFPep resulted in a cyclic peptide with activity comparable to that of the original cyclized molecule and allowed for coupling of this peptide to the affinity column matrix through the free amine group of the side-chain of the lysine. Active (cAFPep and AFPep) and inactive peptides (scrambled peptide and a peptide in which D-val was substituted for L-val) were coupled to affinity columns and the efficiency of coupling evaluated. These peptide affinity columns were utilized to retain proteins from human breast cancer cells which had an affinity for the AFP-derived peptides.

2. ***Development of an affinity chromatography method to isolate cellular binding proteins.*** During the first year, MCF-7 human breast cancer cells were solubilized and passed through the affinity columns. The conditions for solubilization of these cells were optimized and a 70 kDa protein which was retained by both biologically-active and -inactive peptide columns, but not by a blank column, was isolated using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As described in the report from May 2003, the specificity of the binding of this 70 kDa protein for the peptide columns was further evaluated.
- B. Purification and Characterization of Cell-Membrane Binding Protein(s):**
1. ***Identification of a non-specific cellular binding protein, mortalin, isolated by 2D electrophoresis.*** As described in the report for May 2004, to further evaluate the specificity of the retention of the 70 kDa protein by the AFPep column, 2 dimensional (2D) SDS-PAGE was performed. Multiple spots with an approximate molecular weight of 70 kDa were seen using 2D. The most prominent spot isolated was identified by mass spectrometric (MS) analysis as mortalin. This protein was retained by both active and inactive peptides coupled to the affinity columns and was seen in eluents of these columns using both MCF-7 and T47D human breast cancer cells.
 2. ***Identification of a qualitatively-specific cellular binding protein, HSP70, by 1D electrophoresis.*** During the second year (reported in May 2004), 1D electrophoresis was utilized to further probe the specificity of binding of various cellular proteins for the active and inactive peptides. As shown in the May 2004 report, one of the 70 kDa proteins was retained by the active (AFPep) peptide, but not by a control peptide (Figure 1). This band was identified by MS as the inducible form of HSP70 (HSP72). During the third year, this qualitative specificity was further evaluated using Western blotting which has higher sensitivity and specificity than Coomassie staining of an SDS-PAGE. Eluents from active and inactive (control) affinity columns were blotted for inducible HSP70 (HSP72), constitutively-expressed HSP70 (HSP73, HSC70), and ER α . As shown in Figure 2, both inducible and constitutively-expressed forms of HSP70 were retained by the active (AFPep) peptide and by a scrambled peptide which had no biological activity, but substantially less of these proteins were retained by a D-val control column which had the same sequence as that of AFPep, but with a D-val substituted for L-val which renders the peptide inactive. ER α was not retained by any of the peptide affinity columns indicating that the peptide does not bind to ER α (data not shown). Preliminary experiments are currently underway to further examine the importance of the interaction between the AFP-derived peptide and HSP70. Evaluation of the ER α -HSP complex in the presence and absence of peptide by immunoprecipitation is currently underway. (See May 2004 report for a discussion of ER α -HSP heterocomplex).
 3. ***Examination of commercially-obtained AFP receptor binding to AFPep affinity column.*** During the second year (May 2004 report), a commercially-obtained AFP receptor (AFPR) protein and an antibody to this protein were utilized to examine the interaction between AFPR and the AFP-derived

peptide. As described in the May 2004 report, this AFPR was not present in solubilized MCF-7 cells, the affinity chromatography column eluents from these cells, solubilized tumor preparations, or eluents from an AFPeP affinity column through which the solubilized tumor preparation was passed. Furthermore, the AFPR protein was not retained by an AFPeP affinity chromatography column when diluted in solubilization buffer and passed through the column.

4. ***Microscopy to determine subcellular localization of cAFPeP using Alexa 488-labeled peptide.*** During the second year, cAFPeP was coupled to the Alexa 488 fluorophore and the biological activity of the conjugate determined (see report from May 2004). After extensive consultation with investigators who routinely perform radiolabeled drug studies, calculations, and analysis it was determined that radiolabeling of the peptide by inclusion of tritiated Fmoc-valine during peptide synthesis would produce a peptide with a specific activity that would be too low for used in binding and subcellular localization studies. Therefore, during the third year, the Alexa 488-conjugated peptide was utilized as a tool to evaluate internalization of the peptide by human breast cancer cells. However, the fluorescence intensity of this labeled peptide was also below adequate specific activity to visualize binding of the peptide to the cells.

C. Other Research Accomplishments Not Originally Detailed in the Statement of Work:

1. ***Development of an in vitro method to provide a system in which to evaluate the growth-inhibitory activity of cAFPeP.*** During the second year, an *in vitro* assay to measure inhibition by the AFP-derived peptides of the E₂-dependent growth of human breast cancer cells was developed and validated. As described in the May 2004 report, this methodology utilizes tissue culture ware coated with the extracellular matrix (ECM) components fibronectin, collagen I, or collagen IV. This was a seminal discovery because previous studies in our laboratory had shown that breast cancer cells grown on plastic were relatively insensitive to the growth inhibitory effects of cAFPeP. Thus this discovery invokes a role for the ECM in the action of the peptide and provides a simple system to examine the growth-inhibitory activity of the AFP-derived peptides. Furthermore, cells grown in culture under conditions where the peptide inhibited E₂-dependent growth were used to evaluate the modulation of intracellular signaling affected by cAFPeP. The cell culture assay provides a stable endpoint, inhibition of E₂-dependent cellular proliferation, for reference during subsequent signaling studies.
2. ***Investigations of the Modulation of ER α :*** Investigations during the third year focused on using these *in vitro* conditions to study modulation of the ER by treatment with cAFPeP. T47D human breast cancer cells were cultured on ECM components and treated with cAFPeP at 1 μ M followed 35 minutes later by E₂ at 0.1 nM. Control groups received cAFPeP or E₂ alone, or medium only (no-treatment). At various time point up to 24 hours, the cells were harvested, lysed, and immunoblotted. Changes in phosphorylation of three E₂-inducible phosphorylation sites on ER α (serines 118, 104/106, and 167) were examined. Phosphorylation of the putative MAPK phosphorylation site on ER α , serine 118 (Ser118), at 24 hrs. was 3.3 fold higher in the E₂-only group compared to the no-treatment group (Figure 3A). cAFPeP alone resulted in no change in phosphorylation of ER α as compared to the no-treatment control. However, combined treatment with cAFPeP and E₂ resulted in a 55% reduction in the E₂-stimulated phosphorylation of Ser118 at 24 hrs. E₂-induced stimulation of phosphorylation of Ser118 began as early as 30 minutes with seemingly no detectable interference by cAFPeP. But by one hour, cAFPeP had reduced E₂-stimulated phosphorylation by 19% and by 24 hours the level of phosphorylated ER α was substantially reduced by cAFPeP. This suggests that although phosphorylation caused by E₂ is a rapid event beginning by 30 minutes, reduction in phosphorylation by cAFPeP seems to be a cumulative event which is demonstrable at times later than 30 minutes. No consistent change was

seen in the cAFPep-only group at the various time points. Phosphorylation of serine 167 (Ser167) and serines 104 and 106 (Ser104/106) were also evaluated under the same conditions, but at 24 hours, phosphorylation of these sites was not appreciably changed by treatment with E₂ or cAFPep combined with E₂ compared to the no treatment control. These data show that E₂-induced phosphorylation of the putative MAPK (Erk 1/2) phosphorylation site, Ser118, is markedly altered by treatment with cAFPep and suggests that cAFPep in part mediates its effects on the ER by modulating the activity of an upstream component of estrogen receptor signaling that communicates with the ER through MAPK.

Modulation of total cellular levels of ER α were also examined by immunoblot. As shown in Figure 3B, treatment of T47D cells with E₂ resulted in an approximately 23% reduction in the total ER α in the cell at 24 hrs. after treatment. Treatment with cAFPep (1 μ M) significantly reduced the total amount of ER α to 57% of the amount in the no-treatment control ($p < 0.01$). Interestingly, when T47D cells were treated with the combination of cAFPep and E₂, the total ER α was reduced to 12% ($p < 0.001$) of that of cells which received medium alone, which is equivalent to only 16% of the ER that remains with treatment with E₂ only and this reduction as compared to the E₂ treatment group is significant ($p < 0.01$). This suggests that while cAFPep can somewhat reduce the total amount of estrogen receptor in the cell, when combined with E₂, the reduction is substantially magnified perhaps by downregulation and/or degradation of the ER. The reduction in the total ER suggested that the predominant mechanism of action of the AFP-derived peptide is to down-regulate this receptor. Furthermore, combined treatment of T47D cells with cAFPep and E₂ reduced total progesterone receptor-A (Figure 2C). At 24 hours, a slight (10%) increase in PgR protein was seen with treatment with E₂ alone. However, there was a 47% reduction in total PgR in the cell as a result of combined cAFPep and E₂ treatment as compared to the no treatment control and a 50% reduction compared to the E₂-only control group. Taken together, these data indicate that cAFPep interferes with ER activity by affecting both the phosphorylation of the ER, total levels of ER, and transcriptional activity of the ER. Further studies to examine the mechanism of reduction of ER α levels are underway. A manuscript is being prepared to report these findings.

3. ***Preliminary studies of cAFPep-induced changes in signal transduction.*** Begun in the second year, phospho-protein screening of lysates of T47D human breast cancer cells cultured on ECM-coated plates and treated with cAFPep and estradiol was performed. As described in the May 2004 report, the phosphorylation status of various intracellular signaling molecules was affected by treatment with cAFPep alone or combined with E₂. Investigations during the third year followed up on these observations by further probing modulations of numerous signal transduction molecules using immunoblot for total and phosphorylated forms. As shown in Figure 4, phosphorylation of the kinase-activating site (threonine 108/182) of p38- α was substantially increased at 30 minutes by combined treatment with cAFPep and E₂. Phosphorylation of the kinase-activating site of SAPK/JNK showed a similar increase in phosphorylation at 30 min. as well. Substantial changes in phosphorylation with combined treatment with cAFPep and E₂ at early time points were also seen for FAK (Figure 5) and Shc (Figure 6). This data suggests that cAFPep interferes with upstream pathways that link E₂ to pro-growth responses and that this may affect the activity of ER α , and these signal transduction pathways require further study to understand their role in the mechanism of action of cAFPep.

3. Key Research Accomplishments

Year 1

- Synthesized cAFPeP and AFPeP peptides with and without linker for use in affinity chromatography. Synthesized peptides for use in specificity (blocking) experiments and analogs for experiments to determine the pharmacophore. Designed and synthesized peptides to be used as controls for the affinity chromatography procedure. The biological activity of all peptides was evaluated using the estrogen-stimulated immature mouse uterine growth assay. Only peptides that had the anticipated biological activity were used in the affinity chromatography experiments.
- Peptides were linked to the affinity chromatography matrix using the manufacturer's procedure. Coupling was evaluated using HPLC, Kaiser test, and amino acid analysis.
- MCF-7 and T47D human breast cancer cells were cultured and the confluent monolayers solubilized in a buffer containing a non-ionic detergent. The solubilization buffer components were optimized to reduce degradation and promote stabilization of the binding protein/ligand interaction.
- The affinity chromatography procedure was further optimized to increase sensitivity and to allow for elution of highly retained proteins by the use of sequential elutions.
- Proteins retained by the affinity columns were isolated using SDS-PAGE with Coomassie Blue staining to visualize the proteins.
- Control peptides, blocking, and peptide elution experiments were performed to evaluate the specificity of the binding proteins isolated.

Year 2

- Completed optimization of affinity chromatography method to isolate cellular binding proteins for the AFP-derived peptides.
- Used 2D electrophoresis to evaluate the proteins that were highly retained by the AFPeP and Scrambled (control) affinity columns. The most prominent spot (non-specific) was sequenced by Mass Spectrometry (MS) and identified as mortalin.
- Separated the highly-retained proteins from the AFPeP and Scrambled affinity columns by 1D electrophoresis. Isolated a protein which appeared to bind specifically to AFPeP, but not to the control column. This protein was identified by MS as Heat shock protein 70.
- Determined that AFPR protein was not retained by AFPeP affinity column.
- Developed and optimized an *in vitro* method for evaluating the activity of the AFP-derived peptides cultured on extracellular matrix (ECM) components.
- Performed a phospho-protein screening of T47D human breast cancer cells cultured on ECM and treated with estradiol, peptide, and the combination.
- Labeled cAFPeP and AFPeP with Alexa 488 fluorescent dye and evaluated their biological activities.

Year 3

- Determined the quantitative specificity of HSP70 family members (inducible and constitutively-expressed HSP70) for AFP-derived peptide.
- Demonstrated that ER α is not retained by the AFPeP affinity chromatography column.
- Determined that total ER α protein levels in T47D cells are significantly decreased by combined treatment with cAFPeP and E₂.
- Examined the changes in phosphorylation status of ER α at various serine sites with treatment with cAFPeP and cAFPeP combined with E₂.

- Determined that E₂-induced expression of progesterone receptor was substantially decreased by treatment with cAFPeP.
- Followed-up on changes in phosphorylation in response to cAFPeP treatment of various signal transduction molecules that were identified by phospho-protein screen. Molecules that were further probed by immunoblot include p38- α , SAPK/JNK, FAK, and Shc. Substantial changes in phosphorylation with combined treatment with cAFPeP and E₂ were seen at early time points for all of these molecules.

4. Reportable Outcomes

Year 1

- First author on an abstract for presentation at the 2003 Annual Meeting of the AACR entitled "Synthetic peptide derived from alpha-fetoprotein inhibits growth of human breast cancer: Identification of the pharmacophore."
- Co-author on two abstracts for presentation at the 2003 Annual Meeting of the AACR entitled "Molecular mechanisms of alphafetoprotein peptide-associated estrogen receptor activity and the role of mitogen activated protein kinase pathways" and "A peptide derived from the active site of alpha-fetoprotein prevents breast cancer in a rat model."
- Award of a Susan G. Komen Breast Cancer Foundation grant in the amount of \$30,000 over two years to provide funds for supplies and travel monies (no stipend or tuition support) for the project entitled "Identification of Cellular Binding Sites for a Novel Human Anti-Breast Cancer Peptide."
- Received an Inglenook Scholar-in-Training Award to be utilized for travel expenses to attend the 2003 Annual Meeting of the AACR.
- Progress of this research project has been reported at departmental colloquia and at Ph.D. thesis committee meetings.
- This research was presented as a poster at the Albany Medical College Student Research Day. The poster entitled "Identification of Cellular Binding Sites for a Novel Human Anti-Breast Cancer Peptide" was selected as among the six best of the approximately 70 posters presented and consequently the research was presented as a talk at the Albany Medical College Awards Day and was awarded the Dean's Certificate of Excellence in Research.

Year 2:

- First author on a peer-reviewed publication . *J. Peptide Res.*, 2004. 63. 409-419 (10).
- Presented a poster at the 2003 Annual Meeting of the AACR entitled "Synthetic peptide derived from alpha-fetoprotein inhibits growth of human breast cancer: Identification of the pharmacophore." Received an Inglenook Scholar-in-Training Award to be utilized for travel expenses to attend this meeting.
- Received M.S. degree from Albany Medical College after successful completion of all requirements.
- Progress of this research project was reported at a departmental colloquia and Ph.D. thesis committee meetings.
- This research was presented as a poster at the Albany Medical College Student Research Day and was awarded the Dean's Prize for Extramural Research Activities at the Albany Medical College Student Awards Day.

Year 3:

- Progress of this research project has been reported at a departmental colloquia and Ph.D. thesis committee meetings.
- This research was presented as a poster at the Albany Medical College Center for Immunology and Microbial Disease Research Retreat. The poster was entitled “Inhibition by cAFPep of estrogen-dependent growth of T47D human breast cancer cells is facilitated by adhesion to specific extracellular matrix components.”
- This research was presented at the Albany Medical College Student Research Day as a poster entitled “Inhibition of breast cancer growth by AFP-derived peptide: Study of mechanism.”
- Began preparation of a manuscript to report the outcomes of ER modulation studies.
- Presented final data to the thesis committee.
- Completed Ph.D. thesis entitled “Binding sites and Mechanistic Pathways for a Novel Human Anti-breast Cancer Peptide”. Subsequent to this reporting period (May 2005), the Ph.D. thesis was publicly and privately defended, and Ms. DeFreest was awarded her Ph.D. from Albany Medical College.
- Preparing a poster for presentation at the Fourth Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program.

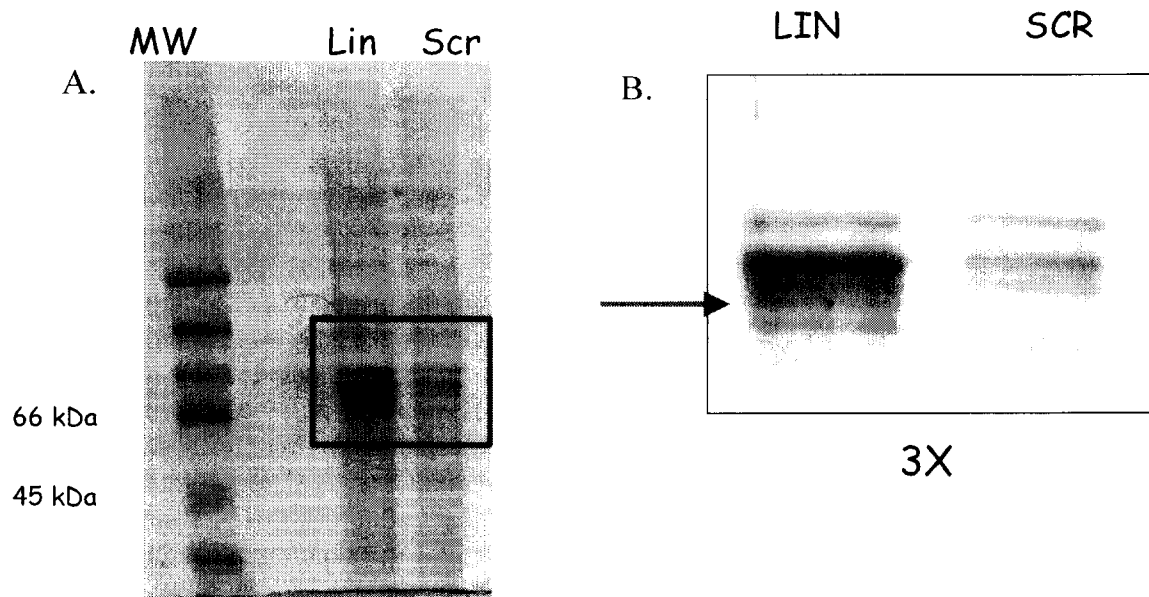


Figure 1: 3 M NaSCN eluents from AFPep(Lin) and Scrambled Control (Scr) columns separated on a 7.5% SDS-PA gel and visualized by Coomassie Blue staining. The specific band of approximate molecular weight 70 kDa is denoted by the arrow in B. This band has been identified by mass spectrometry as inducible heat shock protein 70.

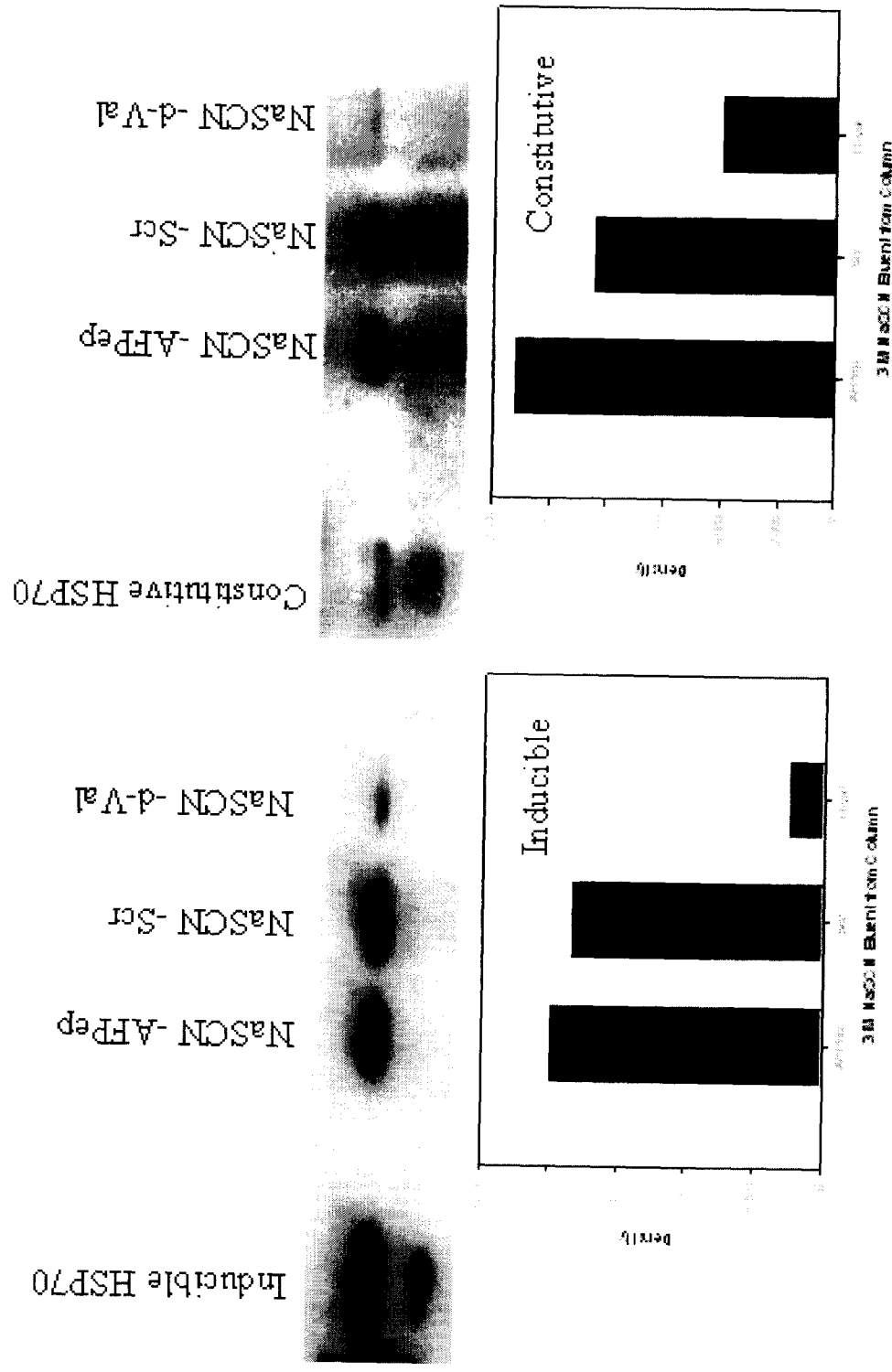


Figure 2: D-val control peptide column retains less inducible and constitutively-expressed HSP70 than affinity chromatography columns with active (AFPep) or scrambled (Scr) peptide. Eluents from T47D human breast cancer cells were immunoblotted for both forms of HSP70. Inducible and constitutively-expressed HSP70 proteins were run concurrently as controls.

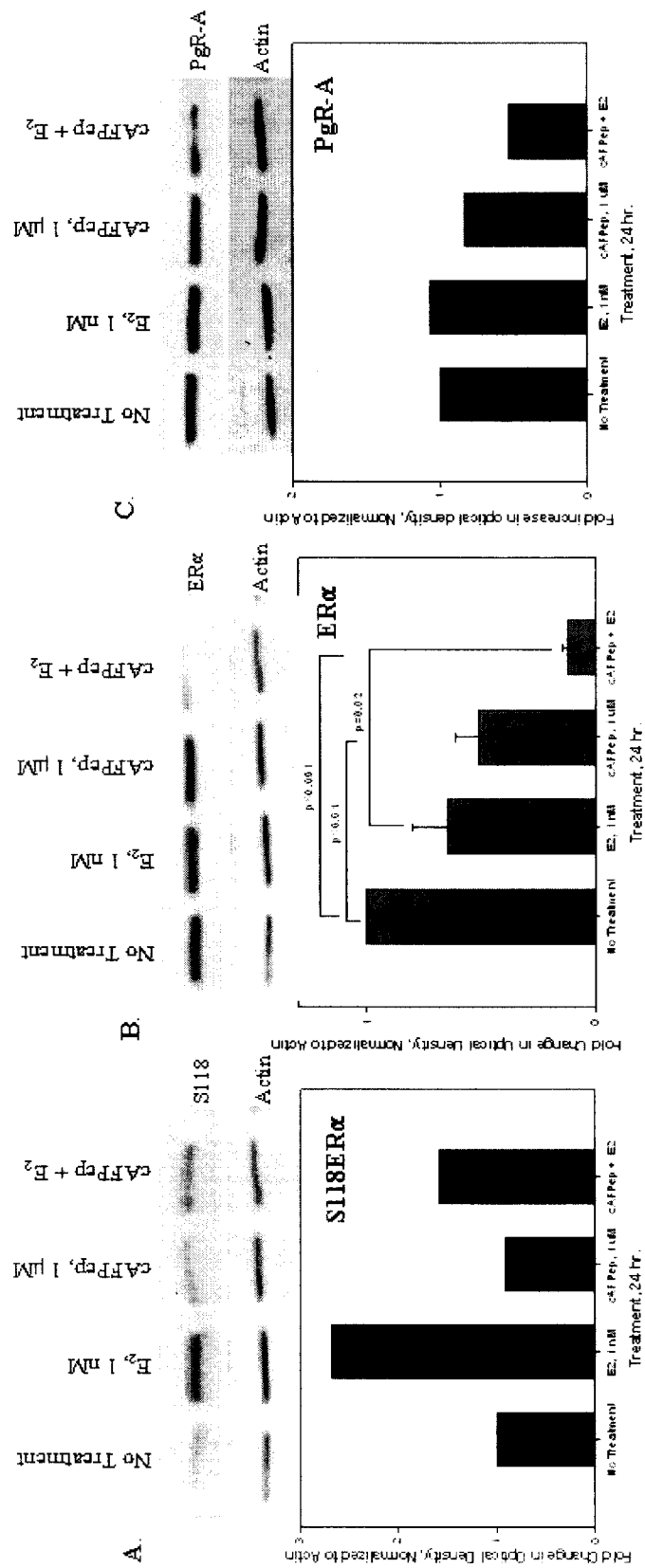


Figure 3: Modulation of ER α phosphorylation, total ER, and total PgR in T47D human breast cancer cells. T47D total cell lysates were treated with E₂ or cAFPep alone, cAFPep combined with E₂, or received no treatment. At 24 hours the cells were harvested, lysed, and immunoblotted using antibodies specific to Ser 118ER α , ER α (total) or progesterone receptor -A (PgR-A). A. cAFPep reduces E₂-induced phosphorylation of serine 118 of ER α at 24 hours. B. cAFPep treatment significantly decreases total ER α both alone and combined with E₂ treatment. C. Combined treatment with cAFPep and E₂ reduces total PgR-A.

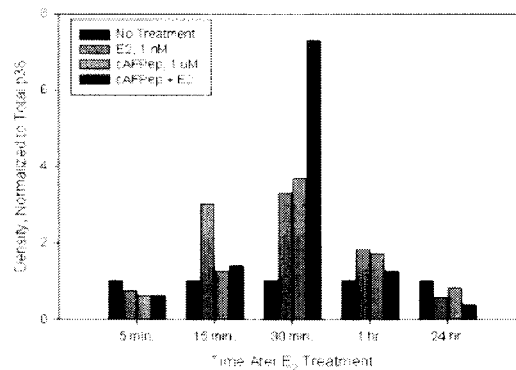


Figure 4: Phosphorylation of the kinase-activating site threonine 180/182 of p38- α . cAFPeP and E₂ combined treatment resulted in a substantial (7-fold) increase in phosphorylation of this site at 30 min.

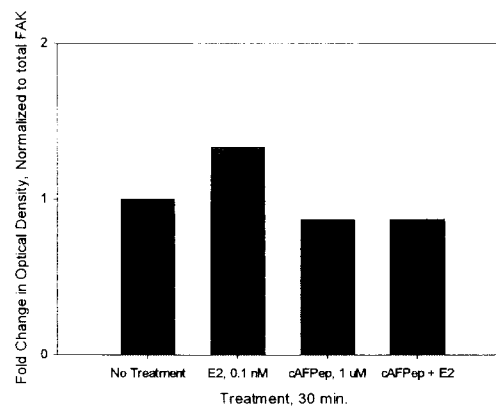


Figure 5: Phosphorylation of the dual kinase-activating site tyrosine 576/577 of FAK. Tyrosine phosphorylation is increased by treatment with E₂ by 33%, but was reduced by combined treatment with cAFPeP and E₂ as compared to both the no treatment and E₂ only groups.

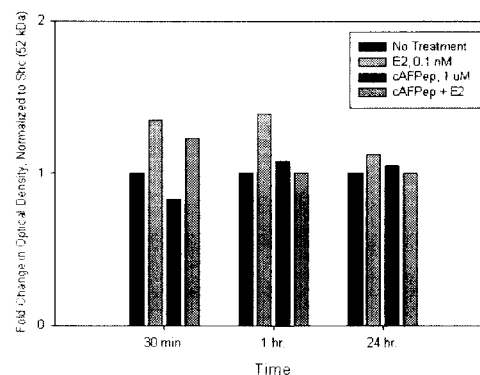


Figure 6: Phosphorylation of the dual kinase-activating site tyrosine 239/240 of the 52 kDa isoform of Shc. At 1 hour, E₂ increases phosphorylation of this site by 39%, but combined treatment with cAFPeP and E₂ reduces phosphorylation as compared to E₂ alone.

5. Conclusions

The work thus far indicates that this novel human anti-breast cancer peptide interferes with estrogen receptor signaling in human breast cancer cells by decreasing total cellular levels of the estrogen receptor and by interfering with upstream signaling pathways that affect the activity of the estrogen receptor. cAFPep interacts with members of the heat shock protein 70 family of proteins and may interfere with the ER α -HSP complex leading to downregulation of ER α expression and activity. In addition, the pharmacophore of the peptide has been determined and an *in vitro* method to measure the activity of the AFP-derived peptides has been developed and validated. Overall, a substantial amount of work has been done to expand the understanding of the mechanism of action of these peptides.

2Taken together, these data suggest a mechanism of action of cAFPep that downregulates the ER by altering the interaction of ER with HSPs. The action of this peptide interferes with ER in a way that is unlike that of any other drug currently used to treat ER+ breast cancer. Further understanding of the nature of the interactions with HSPs to alter the metabolism of the estrogen receptor will shed light on a novel pharmacological interference with estrogen particularly as it relates to prevention of estrogen-stimulated breast cancer growth.

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7. **Appendix** — *J. Peptide Res.* 2004, 63:409-419.

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Synthetic peptide derived from α -fetoprotein inhibits growth of human breast cancer: investigation of the pharmacophore and synthesis optimization

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Key words: breast cancer; estrogen; α -fetoprotein; head-to-tail cyclization; synthetic peptide

Abstract: A synthetic peptide that inhibits the growth of estrogen receptor positive (ER+) human breast cancers, growing as xenografts in mice, has been reported. The cyclic 9-mer peptide, cyclo[EMTOVNOGQ], is derived from α -fetoprotein (AFP), a safe, naturally occurring human protein produced during pregnancy, which itself has anti-estrogenic and anti-breast cancer activity. To determine the pharmacophore of the peptide, a series of analogs was prepared using solid-phase peptide synthesis. Analogs were screened in a 1-day bioassay, which assessed their ability to inhibit the estrogen-stimulated growth of uterus in immature mice. Deletion of glutamic acid, Glu1, abolished activity of the peptide, but glutamine (Gln) or asparagine (Asn) could be substituted for Glu1 without loss of activity. Methionine (Met2) was replaced with lysine (Lys) or tyrosine (Tyr) with retention of activity. Substitution of Lys for Met2 in the cyclic molecule resulted in a compound with activity comparable with the Met2-containing cyclic molecule, but with a greater than twofold increase in purity and corresponding increase in yield. This Lys analog demonstrated anti-breast cancer activity equivalent to that of the original Met-containing peptide. Therefore, Met2 is not essential for biologic activity and substitution of Lys is synthetically advantageous. Threonine (Thr3) is a nonessential site, and can be substituted with serine (Ser), valine (Val), or alanine (Ala) without significant loss of activity. Hydroxyproline (Hyp), substituted in place of the naturally occurring prolines (Pro4, Pro7), allowed retention of activity and increased stability of the peptide during storage. Replacement of the first Pro (Pro4) with Ser maintains the activity of the peptide, but substitution of Ser for the second Pro (Pro7) abolishes the activity of the peptide. This suggests that the imino acid at residue 7 is important for conformation of the peptide, and the backbone atoms are part of the pharmacophore, but Pro4 is not essential.

Valine (Val5) can be substituted only with branched-chain amino acids (isoleucine, leucine or Thr); replacement by D-valine or Ala resulted in loss of biologic activity. Thus, for this site, the bulky branched side chain is essential. Asparagine (Asn6) is essential for activity. Substitution with Gln or aspartic acid (Asp), resulted in reduction of biologic activity. Removal of glycine (Gly8) resulted in a loss of activity but nonconservative substitutions can be made at this site without a loss of activity indicating that it is not part of the pharmacophore. Cyclization of the peptide is facilitated by addition of Gln9, but this residue does not occur in AFP nor is it necessary for activity. Gln9 can be replaced with Asn, resulting in a molecule with similar activity. These data indicate that the pharmacophore of the peptide includes side chains of Val5 and Asn6 and backbone atoms contributed by Thr3, Val5, Asn6, Hyp7 and Gly8. Met2 and Gln9 can be modified or replaced. Glu1 can be replaced with charged amino acids, and is not likely to be part of the binding site of the peptide. The results of this study provide information that will be helpful in the rational modification of *cyclo*[EMTOVNOGQ] to yield peptide analogs and peptidomimetics with advantages in synthesis, pharmacologic properties, and biologic activity.

Abbreviations: AFP, α -fetoprotein; E₂, 17 β -estradiol; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, 1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high-pressure liquid chromatography.

Introduction

Small molecule analogs of proteins, intended for pharmaceutical or other uses, are usually intended to mimic a binding site or active site of the protein, and to provide some or all of the function of the parent molecule. After identifying molecules small enough to be synthetically economical and yet large enough to contain information adequate to provide the desired activity, it is often necessary to optimize the newly developed small molecule in terms of several additional parameters, perhaps including conformational flexibility, potency, storage stability or stability in vivo, or even other parameters that arise during the development process, such as targeting to one of several available receptors. Simultaneous optimization of several of these parameters might be achieved through library screening or combinatorial synthesis approaches. Alternatively, and perhaps especially for situations in which unexpected observations contribute importantly to the developmental process, rational design approaches offer unique opportunities to optimize the structure and function of the small molecule protein analog. Furthermore, explo-

rations of the structure-activity relationships during rational or iterative design and synthesis activities would be expected to contribute to the understanding of the biology and mechanism by which the novel analogs function.

A peptide that inhibits the growth of estrogen receptor positive (ER+) human breast cancers, growing as xenografts in immune-deficient mice has been synthesized (1). *cyclo*[EMTOVNOGQ] was derived from α -fetoprotein (AFP), a safe, naturally occurring human protein produced during pregnancy, which itself has anti-estrogenic and anti-breast cancer activity (2). The peptide was identified by systematically parsing the AFP molecule until it was demonstrated that the oncostatic activity was localized to an eight amino acid sequence (amino acids 472-479) in domain IIIB of the AFP molecule (1,3,4). Mesfin *et al.* synthesized the linear form of the peptide, EMTFVNPG, and showed that it was the minimal sequence necessary to maintain oncostatic activity against breast cancer cells growing as a monolayer in culture or as a xenograft in immune-deficient mice (4). Substitution of hydroxyproline (Hyp, O) for both prolines resulted in a molecule that showed comparable activity and increased storage stability (1). Head-to-tail cyclization of the molecule to yield *cyclo*[EMTOVNOGQ] was facilitated by addition of Gln to the C-terminus of the octapeptide, and it was demonstrated that this cyclic molecule had an active dose range broader than the linear form of the molecule, and peptide stability and shelf life were not compromised (1). The use of a 'head-to-tail' peptide bond, rather than the more usual disulfide bond, may be advantageous for purposes of shelf life, retention of structure and activity in vitro, and elimination of dimers, trimers, and higher-order aggregates that can sometimes develop when disulfide-bonded peptides are stored or used in conditions that do not carefully control the redox state. The method of cyclization that was used was developed by Kates *et al.* (5,6) and is straightforward and expedient.

Although the anti-breast cancer activity of the linear and cyclic AFP-derived peptides is well-documented, the pharmacophore of the peptide has not been elucidated. Consequently, we designed and synthesized a number of analogs of the linear and cyclized peptides to ascertain which atoms are crucial for activity. Because of the relatively few amino acids to be studied and the availability of interspecies homology data, a rational, rather than a combinatorial, approach to substitution was utilized. Conservative and nonconservative substitutions were made for each amino acid as appropriate. Biologic activity data from the analogs were used to determine the

pharmacophore and auxiliary portions of the molecule. Simultaneously, comparisons of synthetic outcomes yielded information that led to improved approaches for synthesizing these peptides. Peptides with key substitutions were produced with high purity, synthetic yield, and biologic activity. The lead analog, *cyclo*[EKTOVNOGN], was demonstrated to inhibit the estrogen-stimulated growth of human breast cancer cells growing as xenografts in immune-deficient mice.

Experimental Procedures

Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). The amino acids used for synthesis included Fmoc-Glu(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Hyp(tBu)-OH, Fmoc-L-Val-OH, Fmoc-D-Val-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gly-OH, Fmoc-Met-OH, Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Tyr(tBu)-OH, and the carboxyl-protected amino acids Fmoc-Glu(OAll), and Fmoc-Asp(OAll). Reagents for peptide synthesis including 1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), N-hydroxybenzotriazole (HOBt), Fmoc-PAL-PEG-PS resin, 20% piperidine in dimethylformamide (DMF), and diisopropylethylamine (DIPEA) were obtained from PerSeptive Biosystems, Inc. (Framingham, MA, USA). Trifluoroacetic acid (TFA), 2-propanol, anisole, ethane dithiol (EDT), tetrakis triphenylphosphine palladium(0) [Pd(PPh₃)], 4-methylmorpholine, DMF, and 17 β -estradiol (E₂) were purchased from Sigma-Aldrich (St Louis, MO, USA). Diethyl ether, ethylacetate, acetic acid, chloroform, and sodium tetra ethylenediaminetetraacetic acid (EDTA) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Dulbecco's modified eagle medium (DMEM), crystalline bovine insulin, MEM nonessential amino acids, penicillin/streptomycin, L-glutamine, and trypsin were obtained from GIBCO/BRL (Gaithersburg, MD, USA) and Cosmic Calf serum was purchased from Hyclone (Logan, UT, USA).

Peptide synthesis

Peptides were prepared using Fmoc solid-phase synthesis as previously described (1,4). A Pioneer Peptide Synthesis system (PerSeptive Biosystems, Inc.) was used to assem-

ble the growing peptide chain on Fmoc-PAL-PEG-PS resin beginning with the C-terminus using N $^{\alpha}$ -protected amino acids. Activation of the C-terminus of incoming amino acids was accomplished by treatment with HATU and DIPEA. Following synthesis, linear peptide was washed with isopropanol and cleaved from the resin by incubation for approximately 4 h in 9.5 mL of TFA : anisole : EDT (90 : 2 : 3) per 0.1 mmol of peptide (0.5 g resin) and side-chain protective groups were removed concurrently. Peptide was precipitated by addition of cold (-20 °C) diethyl ether and washed by repeated extraction with diethyl ether and then with ethylacetate/diethyl ether (1.5 : 1). The peptide was dissolved in a volume of deionized water to achieve a concentration of 5–10 mg/mL and lyophilized.

Cyclization of peptides

Cyclization of peptides was accomplished using the method of Kates *et al.* (5,6). An additional residue, Fmoc-Glu(OAll) or Fmoc-Asp(OAll), was included at the C-terminus during synthesis to facilitate this process. This amino acid was coupled to the resin through the γ -carboxylic acid leaving the α -carboxyl protected with the allyl group. N $^{\alpha}$ -Fmoc deprotection allowed for sequential coupling of the remaining amino acids to the N-terminus. Following synthesis, cyclization of the peptide occurred through a three-step process. First, removal of allyl group from the α -carbonyl of Glu9 was initiated by treatment with 15 mL of a catalyst mixture containing 3 equivalents of Pd(PPh₃) in chloroform/acetic acid/4-methylmorpholine (37 : 2 : 1) per gram of resin for 2 h in the dark. The resin was washed three times with 15 mL of DMF. Secondly, removal of the base-labile N $^{\alpha}$ -Fmoc was accomplished by treatment with piperidine (20% in DMF) followed by washing three times with 15 mL DMF. Finally, activation of the newly deprotected α -carboxyl of Glu9 by overnight treatment of the peptide-resin with HATU : HOBt : DIPEA (3 : 3 : 10 equivalents) in about 8 mL DMF per gram of resin, resulted in the formation of an amide bond between Glu9 and the deprotected-terminus. The peptide resin was washed three times with 15 mL of DMF. After each of the three steps, the peptide resin was evaluated for the presence of free amine groups using the Kaiser test (7). Subsequent cleavage of the cyclized peptide from the resin using the method outlined above resulted in the formation of γ -carboxyamido-derivative of Glu9 or Asp9, that is, Gln9 or Asn9, respectively.

Purification of peptides

Linear peptides were purified using a reverse-phase C₁₈ Sep-Pak cartridge (Waters, Milford, MA, USA). Briefly, a sample containing peptide of unknown purity was loaded onto a pre-washed cartridge and the sample was sequentially eluted with water, 10, 30, and 60% acetonitrile in water. The fraction containing peptide was then lyophilized. Purity of cyclic peptides was determined after separation of the main component from impurities on a Waters gradient semi-preparative reversed-phase liquid chromatographic system. The fraction containing the main peak was collected and lyophilized. Peptides used for structure-activity relationship analysis were purified prior to use in biologic assays, and those used in biologic assays were at 95% or greater purity. Peptides were evaluated by amino acid analysis and mass spectrometry.

Immature mouse uterine growth assay

The anti-estrotrophic activity of peptide was determined using the immature mouse uterine growth assay as described by Bennett *et al.* (2,3). Administration of 0.5 µg of 17β-estradiol (E₂) i.p. to each mouse has been demonstrated to double the uterine weight in 24 h with a corresponding increase in mitotic figures (2,3,8). Swiss/Webster female mice (13–15-day-old, 6–8 g body weight; Taconic Farms, Germantown, NY, USA) were weighed and distributed into treatment groups typically of five mice each such that groups contained mice of comparable weight ranges. Each group received two sequential i.p. injections spaced 1 h apart. The first injection contained test substance or vehicle control. A dose of 1 µg peptide per mouse was used; this is the dose which has been demonstrated to give the maximal inhibitory response for the linear and cyclic hydroxyproline-containing peptides. The second injection contained 0.5 µg E₂ or vehicle. Twenty-two hours after the second injection, mice were weighed and uteri dissected, trimmed free of mesenteries, and immediately weighed. Uterine weights were normalized to body weight (mg uterine per g of body weight) to compensate for differences between body weights of littermates. The inhibition of estrogen-stimulated uterine growth was calculated from the average values for each group using the following equation:

$$\begin{aligned} \% \text{ Growth inhibition} &= 100 \\ &\times (\text{Full E}_2 \text{ stimulation} - \text{E}_2 \text{ stimulation in test group}) \\ &/ (\text{Full E}_2 \text{ stimulation} - \text{No E}_2 \text{ stimulation}) \end{aligned}$$

Human breast cancer xenograft assay

An *in vivo* assay for anti-breast cancer activity was performed using the methodology of Bennett *et al.* (2,9). Confluent MCF-7 human breast cancer cells growing in monolayer in DMEM without phenol red supplemented with 5% bovine calf serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.1 mM nonessential amino acids, and 10 ng/mL bovine insulin were released from the flask using trypsin/EDTA (0.25%/0.25%) and 20 million cells were pelleted by centrifugation at 200 g and then solidified into a fibrin clot by treatment with 10 µL fibrinogen (50 mg/mL) and 10 µL thrombin (50 units/mL). The solid tumor mass was then cut into pieces of approximately 1.5 mm diameter. Each tumor segment was implanted under the kidney capsule of an Institute for Cancer Research (ICR)-severe combined immunodeficient (SCID) male mouse (Taconic Farms) which weighed about 25 g. Estrogen supplementation was accomplished by s.c. placement of a 2 mm silastic tubing implant containing solid E₂ inserted on the day of tumor implantation. Peptide was injected i.p. once daily at a dose of 10 µg/mouse. This is the dose that has been demonstrated to give the maximal inhibitory response for the cyclic hydroxyproline-containing peptide in this assay. Tumor growth was monitored during survival laparotomy at 14 and 20 days after implantation by measurement of the short (*d*) and long (*D*) axes of the tumor by using a dissecting microscope equipped with an ocular micrometer. Tumor volumes were calculated assuming the tumor shape to be an ellipsoid of revolution about the long axis (*D*) using the formula $(\pi/6)d^2D$. Mean tumor volume ± SE was calculated for display in growth curves. All animal care procedures were approved by the Albany Medical College Animal Care and Use Committee.

Homology data

Sequence information was obtained by comparison of the homologous region of AFP for the six species studied using sequence data published in the protein data base of the National Center for Biotechnology Information provided by the National Library of Medicine.

Statistics

Significance of results for the immature mouse uterine growth assay was determined by Dunnett's multivariate

comparison using the 8-amino acid, hydroxyproline-containing linear molecule as the control. There were five to 10 replicates per group except for the Met2Lys and control peptide (8-mer Hyp) where there were 28 and 332 replicates, respectively. Results were considered significant at $P < 0.05$. Error is shown as \pm standard error (SE) of measurement. Significance of differences between groups for the human breast cancer xenograft assay was tested using the one-sided Wilcoxon sum of ranks test. There were five to six mice in each treatment group.

Results

Part I: Identification of the active site of the peptide

To determine which residues were essential to the bioactivity of the 8-amino acid, linear peptide (sequence Glu1-Met2-Thr3-Pro4-Val5-Asn6-Pro7-Gly8), a series of linear analogs was prepared and evaluated using the immature mouse uterine growth assay. It has been documented that there is excellent correlation between the uterine growth assay and the human breast cancer xenograft assay with regard to peptide inhibition of estrogen-stimulated growth (1,3). The uterine growth inhibition assay provided the advantages of faster assay time (1 day vs. 20 days) and substantially lower cost. Hence the xenograft assay was used only for the lead analog. Modifications of peptides included conservative substitutions for each of the amino acids, deletions of the N- and C-termini, and for some amino acids, nonconservative substitutions as well.

Glu1

Replacing the highly conserved Glu1 with Gln or Asn maintained the inhibitory activity of the linear peptide (Fig. 1). Substitution of this residue with Lys or Asp reduced the activity of the molecule, but did not abolish it. However, the nonconservative substitution of Glu1 with Ser resulted in an almost complete loss of the inhibitory activity of the peptide. Deletion of Glu1 ablated the inhibitory activity of the molecule.

Met2

Met2 is not highly conserved across species in AFP (Table 1) and can be replaced with Thr (chimpanzee), Glu (horse), or Ala (mouse, rat). A linear octapeptide was synthesized with a substitution of Lys for Met2. The peptide retained full inhibitory activity (Table 2). Substitution of Tyr for Met2 also maintained the inhibitory activity.

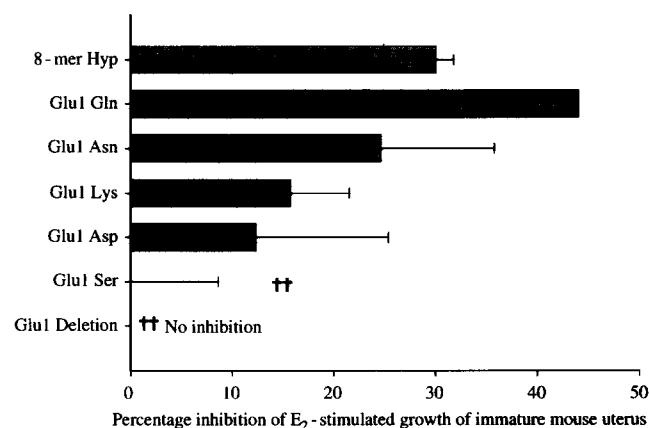


Figure 1. Anti-estrotrophic effect of substitutions for glutamic acid. Glu1 was substituted as shown in 8-amino acid, linear AFP-derived peptide containing substitutions of Hyp (O) for both Pro, EMTOVNOG (8-mer Hyp). Inhibition was measured using the immature mouse uterine growth assay. Peptide at 1 μ g per mouse or vehicle control was injected i.p. into immature Swiss mice. One hour later, the mice were injected i.p. with 0.5 μ g E₂ or vehicle control. Twenty-two hours after the second injection, mice were killed, the uteri dissected and trimmed free of mesenteries, and weighed. Percent inhibition is calculated as described in Experimental Procedures. 8-mer Hyp showed inhibition of growth of approximately 33% in this assay. $^{††}P < 0.005$ compared with 8-mer Hyp using Dunnett's multivariate comparison.

Table 1. Comparison of sequence of AFP and albumin from different species

Residue number	1	2	3	4	5	6	7	8
Human	<i>E</i>	<i>M</i>	<i>T</i>	<i>P</i>	<i>V</i>	<i>N</i>	<i>P</i>	<i>G</i>
Gorilla	<i>E</i>	<i>M</i>	<i>T</i>	<i>P</i>	<i>V</i>	<i>N</i>	<i>P</i>	<i>G</i>
Chimpanzee	<i>E</i>	<i>T</i>	<i>T</i>	<i>P</i>	<i>V</i>	<i>N</i>	<i>P</i>	<i>G</i>
Horse	<i>E</i>	<i>E</i>	<i>S</i>	<i>P</i>	<i>I</i>	<i>N</i>	<i>P</i>	<i>G</i>
Rat	<i>E</i>	<i>A</i>	<i>N</i>	<i>P</i>	<i>V</i>	<i>N</i>	<i>S</i>	<i>G</i>
Mouse	<i>E</i>	<i>A</i>	<i>S</i>	<i>P</i>	<i>V</i>	<i>N</i>	<i>S</i>	<i>G</i>
Albumin (human)	<i>E</i>	<i>K</i>	<i>T</i>	<i>P</i>	<i>V</i>	<i>S</i>	<i>D</i>	<i>R</i>

Amino acids that are in bold italic (Residues 1, 4, 6 and 8) are strictly conserved between species and those in italics are semi-conserved. Residues that are in bold (Residues 2, 3 and 7) are not conserved in AFP across those species studied.

Thr3

Analogues of the peptide with Thr3Val and Thr3Ala substitutions in the 8-mer peptide were prepared and tested (Table 2). Both of these analogues retained the inhibitory activity of the parent molecule. Substitution of Ser for Thr3 resulted in some reduction in inhibitory activity that was not statistically significant.

Pro4,7

The conservation of Pro4 across species (Table 1) suggested that this residue was required for the biologic activity of the

Table 2. Anti-estrotrophic effect of nonconservative substitutions in AFP-derived linear peptide

Nonconservative substitutions: analogs of linear peptides		
Peptide/substitution	Sequence	Relative activity
8-mer Hyp	EMTOVNOG	++
Met2	EKTOVNOG	++
	EYTOVNOG	++
Thr3	EMVOVNOG	++
	EMAOVNOG	++
	EMSOVNOG	+
G8-deleted	EMTPVNP	+
Horse	EESPINPG	+
Horse P4O,P7O	EESOINO	++
Albumin peptide	EKTPVSDR	-

Percent inhibition was measured using the immature mouse uterine growth assay. Amino acid substitutions of the 8-amino acid, linear peptide containing either Pro or Hyp are shown in bold. ++, 25–40% inhibition; +, 12–25% inhibition; –, less than 5% inhibition.

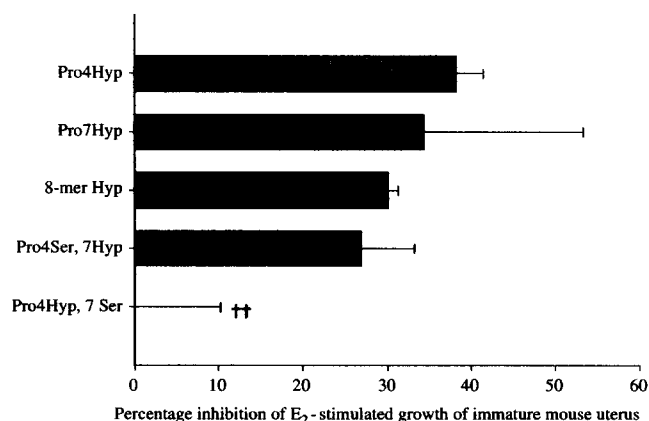


Figure 2. Anti-estrotrophic effect of substitutions for proline in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. $^{**}P < 0.005$ compared with 8-mer Hyp using Dunnett's multivariate comparison.

peptide; Pro7 is less strictly conserved. As shown in Fig. 2, analogs containing substitutions of hydroxyproline (Hyp) for either Pro4 or Pro7 resulted in retention of inhibitory activity. Similarly, an analog with Hyp substituted for both Pro4 and Pro7 retained inhibition. An analog containing the nonconservative substitution of Ser for Pro4 maintained inhibitory activity. However, substitution of Ser for Pro7 resulted in a complete loss of biologic activity (Fig. 2) although Ser replaces Pro7 in the AFP of rodents (Table 1).

Val5

Conservative substitutions for Val5 included Ala, Leu, and Ile, each of which has hydrophobic character. Substitution

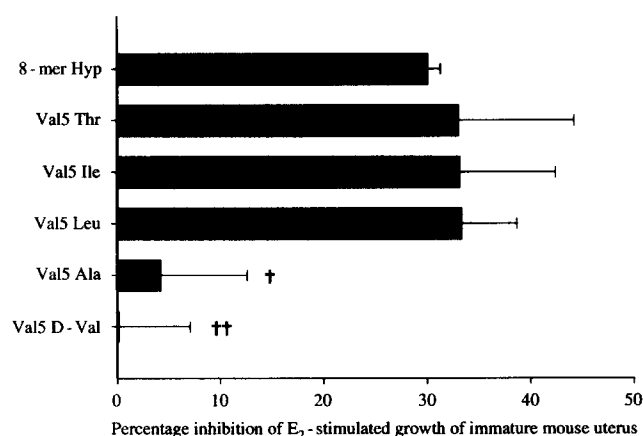


Figure 3. Anti-estrotrophic effect of substitutions for valine in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. $^{\dagger}P < 0.01$; $^{**}P < 0.005$.

of Val5 with Leu, Ile, or Thr maintained biologic activity (Fig. 3). However, replacement of Val5 with Ala resulted in a nearly complete loss of inhibitory activity, and replacement of Val5 with D-Val completely abrogated the activity of the analog.

Asn6

Peptide analogs were synthesized with conservative substitutions for Asn6 and analyzed for inhibitory activity using the immature mouse uterine growth assay. Replacement of Asn6 with Gln resulted in a significant loss of inhibitory activity (Fig. 4). Substitution with the carboxylic acid derivative of this residue, Asp, also resulted in a reduction in activity.

Gly8

Glycine has been noted as essential for the linear molecule because deletion resulted in a loss of inhibitory activity (4).

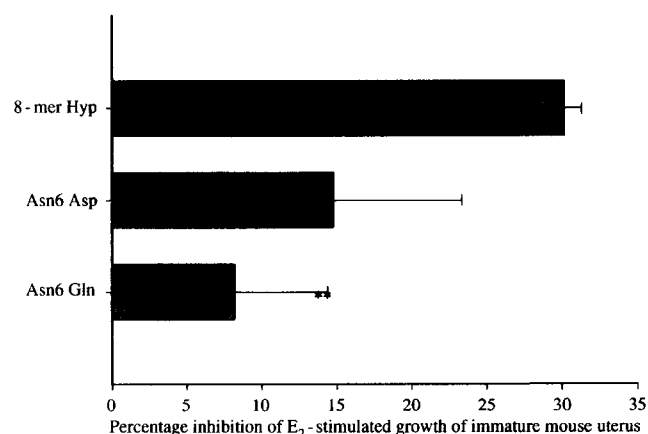


Figure 4. Anti-estrotrophic effect of substitutions for asparagine in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. $^{**}P < 0.05$.

This is consistent with its conservation across species. However, replacing Gly8 with Asn resulted in a peptide that retained full activity (data not shown).

Albumin peptide

AFP is a member of the albumin family of proteins and has a 39% homology of primary structure with albumin (10). This similarity is greatest in the third domain where the AFP-derived peptides were identified. A synthetic peptide with the sequence of human albumin, EKTPVSDR (Table 1), was prepared and analyzed using the immature mouse uterine growth assay (Table 2). This molecule differs from the AFP-derived linear peptide by substitutions of Lys for Met2, Ser for Asn6, Asp for Pro7, and Arg for Gly8. The synthetic albumin peptide had no inhibitory activity.

Part II: Comparison of activity, yield, and purity of cyclic analogs of peptide

Mesfin *et al.* (1) had shown previously that the linear peptide could be cyclized and that the resultant molecule, *cyclo*[EMTOVNOGQ], was anti-estrotrophic and that cyclization of the molecule broadened the effective dose range. Cyclization of the molecule was facilitated by addition of a Gln to the C-terminus of the molecule, and, in the linear form, this addition did not alter biologic activity (1). As the data in Table 2 indicated that Lys could be substituted for Met2 without loss of biologic activity, we added either Gln or Asn to the C-terminus of the Met2Lys analog and cyclized it. These analogs had inhibitory activity comparable to that of the cyclized molecules containing methionine (Table 3). However, when the purity of the Lys-containing molecules was evaluated by HPLC, the chromatograms showed the presence of predominantly one peak. The cyclic Met-containing peptides chromatographed as multiple peaks and required further purification by semi-preparative HPLC. This additional step in processing of the peptides resulted in a significant loss of material. The cyclic peptide containing the Met2Lys substitution increased the purity of the preparation 2.4-fold from 39 to 94% for the Gln-containing analog and fourfold from 24 to 95% for the molecule cyclized using Asn (Table 3). As a consequence, the yield of the peptide was increased compared with the cyclic molecule containing Met.

The cyclized peptide containing the Met2Lys substitution was evaluated for its ability to inhibit the growth of ER+ human breast cancer cells growing as xenografts under the kidney capsule in immune-deficient mice. As shown in

Table 3. Activity, purity, and yield of cyclic analogs of AFP-derived peptide

Analog name	Activity (% inhibition)	Purity (%)	Yield (%)
<i>cyclo</i> [EMTOVNOGQ]	29	39	56
<i>cyclo</i> [EMTOVNOGN]	23	24	62
<i>cyclo</i> [EKTOVNOGQ]	24	94	67
<i>cyclo</i> [EKTOVNOGN]	32	95	72

Cyclization of the 9-amino acid linear peptides was performed as described in Experimental Procedures. Biologic activity was measured in the immature mouse uterine growth assay after final purification of the peptides. Purity was determined as area percent by HPLC prior to purification. Yield was calculated as amount of peptide recovered after lyophilization without further purification by HPLC.

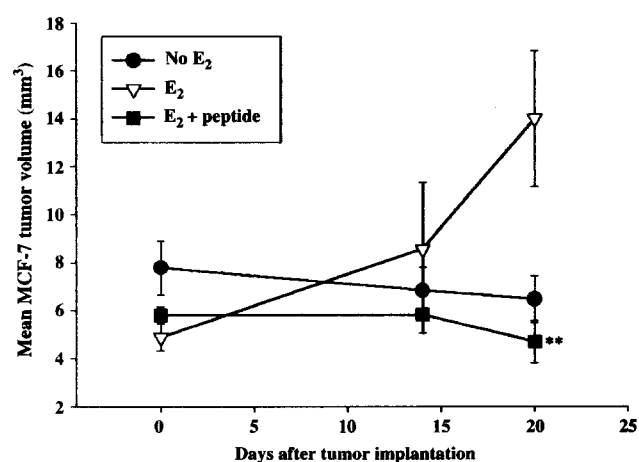


Figure 5. Anti-estrotrophic activity of *cyclo*[EKTOVNOGN] against MCF-7 human breast cancer xenografts. There were five to six mice per treatment group. The assay is described in Experimental Procedures. E₂ was provided by implantation of a silastic tube containing solid E₂ inserted subcutaneously on day 0. Peptide was given once daily at 10 µg/mouse. Peptide significantly inhibited the estrogen-dependent growth of the tumor. ***P* < 0.05.

Fig. 5, growth of MCF-7 tumors was dependent on estrogen for growth: the presence of E₂ resulted in a threefold increase in tumor volume after 20 days in comparison with the control without E₂. Administration of 10 µg *cyclo*[EKTOVNOGN] daily to animals concurrently treated with E₂ prevented tumor growth over this same time period.

Discussion

Rationale for investigation of the pharmacophore of AFP-derived peptides

It had been demonstrated previously that both the linear and cyclic AFP-derived peptides were capable of inhibiting

the growth of estrogen-dependent breast cancers growing as xenografts in immune-deficient mice (1,3). Furthermore, it had been shown that these peptides inhibit ER+ tumors that had been made resistant to tamoxifen (3). Although the mechanism of the oncostatic action of the peptides has not been elucidated, it is known that, unlike tamoxifen or other selective estrogen receptor modulators, the peptides do not compete with estrogen for the ligand-binding domain of the estrogen receptor (3). Rather, it is likely that the peptides mediate their activity by interacting with a cell surface-binding protein, as does their parent molecule AFP, although this receptor has not been fully characterized (11–15). As the receptor for the peptides is unknown, we chose to evaluate the structure–activity relationship of the peptides employing rational design techniques to determine the key amino acids required for maximal biologic activity. Two important biologic endpoints, namely inhibition of estrogen-stimulated growth of uterus and of breast cancer were employed as measures of biologic activity. A combinatorial approach to analog development was not chosen because: (a) comparative sequence data for the oncostatic region of AFP were available for six mammalian species (Table 1) suggesting which residues were essential for activity based on conservation of those residues. Although the oncostatic potential of peptides derived from the AFPs of each of the six species shown in Table 1 was not tested, comparison of the sequences of these peptides served to guide our choices for individual amino acid substitutions when designing analogs; (b) relatively few amino acids (eight) comprise the minimal sequence necessary for anti-estrotrophic activity in AFP-derived peptides; and (c) data generated while determining the minimal sequence and optimizing the stability of the peptides (1,4) suggested key residues that were required for activity or stability in storage and other residues which could be replaced without loss of biologic activity. The unexpected observation that cyclic molecules provided a wider dose–response curve (1) suggested the importance of maintaining the residues necessary for the cyclization process. Thus, the rational approach seemed more appropriate for these studies.

Investigation of the pharmacophore

Cyclization of the peptide as previously described (1) constrains the molecule, causing the peptide to exist largely as a planar macrocycle (Fig. 6). The region of the cyclic molecule near the ring-closing peptide bond (the Asn to Glu link) is probably not the pharmacophore, as that peptide

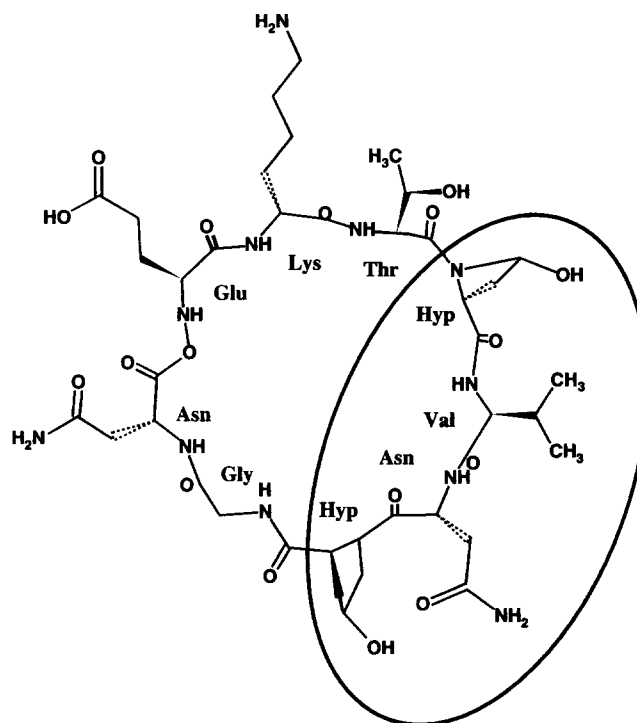


Figure 6. Sequence and structure of *cyclo*[EKTOVNOGN]. Structure of the cyclic nonapeptide. Amino acids are labeled adjacent to their side chains and the pharmacophore is circled.

bond is artificial and does not exist in the parent protein. As such, it is logical to assume that the portion of the molecule diametrically across from that Asn to Glu peptide bond, that is, the 'middle' of the linear peptide, ought to be the pharmacophore. As a first approximation, the region may be postulated to consist of the amino acids between the two hydroxy proline residues (Fig. 6).

In the six species evaluated, Val₅ is found at this site for all species except equine where the highly conserved Ile is found in place of Val₅ (Table 1). Consistent with the presence of Ile in equine AFP, synthetic analogs with Val₅ substitutions, which retained the hydrophobic and branched character, such as Ile and Leu, retained biologic activity. However, replacement of Val₅ with the smaller, nonbranched Ala resulted in a significant loss of inhibitory activity, suggesting that the branched structure of the side chain at this site was required to maintain the activity of the peptide. To test this hypothesis, Thr was substituted for Val₅, a substitution that conserves the branched nature, but is more hydrophilic than Val. This analog retained anti-estrotrophic activity suggesting that the branched nature of the residue at this position is necessary for maximal activity. Interestingly, substitution of Val₅ with its enantiomer, D-Val, completely ablated the activity and suggested that the specific orientation of the Val side chain

or of the backbone participates in the binding of the pharmacophore to its target.

Asn6 is strictly conserved across those species evaluated and even conservative substitutions of Asp or Gln for Asn6 resulted in a loss of inhibitory activity. This strongly suggests that Asn6 is an essential residue of the active site of this molecule, which correlates with what was expected based on the interspecies homology.

As shown in Fig. 2, Hyp can be substituted for either or both proline residues without a loss of biologic activity and it has been demonstrated that Hyp increases the storage stability of the AFP-derived peptides (1). An interspecies comparison of Pro4 and Pro7 indicates that Pro4 is highly conserved, while Pro7 is substituted by Ser in rodents. To examine the requirement for proline at these sites, analogs were prepared which replaced Pro4 or Pro7 with Ser. Counterintuitive to the interspecies comparisons, the inhibitory activity of the molecule was retained when Pro4 was substituted, but completely abolished when Ser replaced Pro7. Thus, the imino acid at residue 7 is important for conformation of the peptide, and the backbone atoms at this site are part of the pharmacophore. The substitution of Ser for Pro4 suggests that Pro4 is not essential.

The pharmacophore of the peptide seems to lie between the two proline residues and includes the side chains of valine and asparagine, and the backbone atoms contributed by valine, asparagine, and Pro7. This region is highly conserved among mammalian species and conservative substitutions in this area led to diminution or loss of biologic activity.

Amino acids distal to the pharmacophore contribute to the conformational stability of the peptide

Glu1 is highly retained across species and this initially suggested that it may be important to the activity of the molecule. Conservative substitutions in the linear, 8-amino acid peptide of Gln or Asn showed no significant loss of activity (Fig. 1). Replacing this residue with Lys, which retains a charge at this site, or the highly conservative substitution of Asp showed some loss of activity, but it was not significant. However, replacing Glu1 with Ser causes a loss of biologic activity and deletion of Glu1 completely ablated the inhibitory activity of the molecule. Since substitutions in the linear molecule which affect both the charge at this site and the size of the side chain resulted in analogs which retained their biologic activity, it is unlikely that Glu1 is part of the binding site of this peptide. Rather it

more likely plays a role in maintaining the conformational stability of the molecule.

Replacing Thr3 with Val, which is more hydrophobic but retains the branched nature of the side chain, maintained the inhibitory activity of the linear molecule (Table 2). The nonconservative substitution of Ala also maintained this activity. Although there was some loss of activity when Ser is substituted, this loss was not significant (data not shown), and considered with the nonconservative Val and Ala substitutions, it can be concluded that the side chain of this residue is not required for biologic activity.

The highly conserved nature of the Gly8 residue suggested that this residue may be required (Table 1). Deletion of Gly8 resulted in a reduction in the biologic activity of the molecule, but did not abolish it (Table 2). Furthermore, the nonconservative substitution of Asn for Gly8 resulted in a molecule that retained full activity. This indicates that Gly8 is not part of the active site of this peptide.

Met2 shows no interspecies homology for the six species evaluated (Table 1) and synthetic analogs in which Met2 was replaced by either tyrosine or lysine in the linear peptide showed no loss of biologic activity. Taken together, the data indicate that Glu1, Met2, Thr3, and Gly8 are not part of the pharmacophore, but it is likely that they contribute to the conformational stability of the molecule because they can be nonconservatively substituted without significant loss of activity. They probably function to maintain the linear molecule in a conformation that is conducive to binding to its receptor. The energy-minimized structure of the 8-amino acid Met-containing peptide has been previously published (4) and this molecule shows the potential to form a horseshoe shaped structure.

The substitution of Lys for Met2 was especially advantageous. Molecular modeling (not shown) indicated that the 8-amino acid molecule containing this substitution maintained the horseshoe structure seen previously with the methionine-containing molecule (4). Cyclization of the linear peptide containing the Met2Lys substitution (Fig. 6) produced a peptide that was far superior in synthetic outcomes with increased purity (Table 3). The optimized analog, *cyclo*[EKTOVNOGN] not only had anti-uterotrophic activity (Table 3) but also had oncostatic activity in that it inhibited the estrogen-stimulated growth of human breast cancer cells growing as xenografts in immune-deficient mice in a dose dependent manner consistent with that of *cyclo*[EMTOVNOGQ] (Fig. 5) (1).

The cyclic peptide is derived from AFP which itself has anti-estrogenic and anti-breast cancer activity. Sonnensch-

ein *et al.* (16) have demonstrated that the growth of an estrogen-dependent breast cancer is inhibited in rats bearing an AFP-secreting hepatoma. Tumor growth inhibition was not found when these investigators used an estrogen-independent breast cancer. Furthermore, Jacobson *et al.* (17) and Richardson *et al.* (18) have shown that elevated levels of AFP during pregnancy are associated with subsequent reduction in the lifetime risk for breast cancer. Jacobson and Janerich (19) have hypothesized that this reduction should be caused by a diminution in estrogen-dependent breast cancers. Bennett *et al.* (2,3) have shown that AFP purified from a human hepatoma culture and then injected into tumor-bearing immune-deficient mice stopped the growth of ER+, but not ER-, human breast cancer xenografts in these mice. Although AFP has demonstrated oncostatic activity (2), it has limited clinical usefulness. It has been shown to aggregate (20) and commercial preparations do not maintain biologic activity. The AFP used for the studies of Bennett *et al.* (2) utilized AFP isolated and purified from a hepatoma culture and the purification procedure

is not trivial. Peptides produced using solid-phase Fmoc synthesis can be produced in high yield and purity in relatively large amounts, and the AFP-derived peptides have shown consistent activity over prolonged storage (1). Generation of a small, stable, biologically active peptide such as described here, and earlier (1,3,4), may suffice for investigations of the mechanism by which the peptide, and of AFP itself, stops the growth of tumor xenografts and exerts its anti-estrotrophic activity. Further, this peptide may be adequate for preclinical investigations as an oncostatic agent, especially if the peptide can be shown to have appropriate pharmacokinetics and low toxicity. At the very least, this cyclic peptide can serve as a model on which to develop peptidomimetics.

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